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Role of Extracellular Proteases in Biofilm Degradation Produced from *Escherichia coli* and *Pseudomonas mendocina*

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A B S T R A C T

One of the most important properties of the protease enzyme is to degrade the biofilm because removal of biofilms is very difficult. In industrial settings, both the inactivation and removal of biofilms are of huge concern. If only disinfection without the removal of attached biofilms occurs, the inactivated biofilm cells may provide an ideal environment for further adhesion and growth, resulting in a complex matrix. Microbial resistance to biocides and their negative environmental impact are the main reasons for finding alternative biofilm control strategies. Enzymes are highly selective and capable of distrupting the structural stability of the biofilm EPS (extracellular polymeric substance) matrix. The biofilms acts as a barrier for antibiotics to affect the pathogenic bacteria and this may account for some people developing chronic Borreliosis/ Lyme symptoms. However proteolytic enzymes are very effective at dissolving mucupolysaccharides in biofilm. The 0.2 ml of purified alkaline protease of molecular weight of 29 kDa has been applied for degrading the biofilm of *Pseudomonas* or *E.coli in* 10 minutes. When biofilm was treated with the thermophilic protease enzyme the growth of bacteria producing biofilm was minimum (*E.coli* 0.230 at $A_{660\ nm}$ and *Pseudomonas mendocina* 0.120 at A_{660nm}). The combination of protease and polysaccharides was successful in biofilm removal or detachment of the organisms.

Introduction

Enzymes played a significant role in several aspects of life from time immemorial. They are in fact vital for the existence of life itself. Enzymes play crucial role in producing the food we eat, the clothes we wear and even in producing fuel for our automobiles. They are important in combating environmental pollution. Leather processing is one of the important industries closely related to everyday life. Proteases are the most important industrial enzymes that execute a wide variety of functions and are thus employed in many important biotechnological applications (Joshi, 2010). They constitute two third of the total enzymes used in various industries and at least a quarter of the total global enzymes production (Banik *et al.*, 2004). These enzymes occupy a pivotal position due to their wide applications in food processing, pharmaceuticals, peptide synthesis

and leather processing (Kalaiarasi *et al.,* 2009) Use of enzymes for industrial processing has received considerable attention in recent years (Ibrahim *et al.,* 2009). *Pseudomonas aeruginosa* and *Escherichia coli* are the most prevalent Gram-negative biofilm forming medical device associated pathogens (Cole *et al.,* 2014). Its presence in food and food environments is not regulated, but because of its good biofilm-producing ability it easily colonizes in pipes, heat-exchangers, air-conditioners, etc. *Pseudomonas aeroginosa* form biofilm in many milk storage tanks and milk storage lines.

Materials and Methods

Sample collection and revival of protease producing organism

Protease producing microorganisms (*Bacillus subtilis*) was collected from Microbiology department of SILB, Solan which was isolated and optimized previously.

Qualitative analysis for the proteolytic activity

Bacillus subtilis was spot inoculated on skim agar and plate was inoculated at 50˚C for 24-48 hrs. After incubation, plates were observed for the clearing zone around the colony which indicated the proteolytic activity of the test bacterial culture.

Quantitative analysis

Protease production was done by using medium containing (g/l) glucose-2.0, casein-0.5, peptone-0.5, and yeast extract-0.5, KH_2PO_4-1.0, $MgSO_4$-0.5 and $FeSO_4.7H_2O$-0.1. Erlenmeyer flasks containing 50ml sterilized medium was inoculated with 5ml suspension of culture. The flasks were incubated at 50 ℃ for 48hrs at 120 rpm in rotary shaker. After incubation, content was centrifuged at 10,000 rpm for 10 min and supernatant was used as a source of enzyme.

Enzyme activity

Protease activity was determined by using skim milk according to the method Leighton *et al.,* 1973. The reaction mixtures containing 250 ml of 1% (w/v) skim milk as a substrate , 250ml of 0.5M Tris buffer pH 9.0 and 250ml of culture supernatant was incubated at 50℃ for 10 min. The reaction was stopped by the addition of 3 ml of 5% (w/v) trichloroacetic acid. Then each tube was centrifuged at 10,000 rpm and the absorbance of the supernatant was taken at 275 nm. One unit of the enzyme activity (U) was equal to the amount of cell free supernatant required to increase absorbance at 275 nm by one unit under the assay condition. Protein content was estimated by method of Lowry by using Bovine serum albumin as the standard.

Protein estimation

The protein estimation was determined by the Lowry method by using bovine serum albumin as a standard (Lowry *et al.,* 1951).

Biofilm production

The standard biofilm producing bacterial strains i.e. *E.coli* (MTCC-1687) and *Pseudomonas mendocina* (MTCC-11808) was procured from the Microbial Type Culture Collection (MTCC), IMTECH Chandigarh. For the biofilm formation the cells were grown in micro titre plate for the desired period of time and then the wells were washed to remove planktonic bacteria. Remaining cells adhered to the wells were subsequently stained with a dye that allows visualization attachment pattern. This surface associated dye was also be solubilized for semi quantitative assessment of the biofilm formed. *Pseudomonas mendocina and E.coli* culture

was inoculated in 3 to 5ml tryptic soya broth and incubated at 37˚C for 24 hrs. This served as inoculum.

Biofilm degradation

The fundamental base for all biofilm studies is the use of appropriate cultivation techniques. Biofilm formation and quantification using glass test tubes was measured. Briefly, overnight cultures of *Pseudomonas mendocina and E.coli*, 200 µl inoculated in biofilm growth medium and incubated overnight at 37˚C. To screen for the efficiency of enzyme in removing the biofilm; the enzyme treatment was given in two ways such as in the medium during the incubation, and after the incubation at 37˚C. After incubation, the growth medium was gently mixed by adding 2ml of cleaning solution (45˚C water was used as control) and incubated the tubes for 30 mins.

After which the solution was gently pipette out. Than 2ml water and 200µl of 0.1% crystal violet solution was added in each tube and incubated for 15mins. Later the tubes were rinsed three times with water. Crystal violet stained biofilms were solubilised in 400µl ethanol and thus formed crystal violet ethanol solutions were measured for absorbance at 595nm.

Results and Discussion

A total of 15 bacterial isolates were obtained (Table 3.1), from soil and water samples. The results depicted in table 3.2 shows that out of 15 isolates only 8 bacterial isolates (ATP-P1, ATP-P3, ATP-P5, ATP-P6, ATP-P7, ATP-P11, ATP-P12, and ATP-P14) hydrolyzed the casein (Figure 3.1). The maximum casein hydrolysis was observed with a bacterial isolate ATP-P5 In the research work. The maximum casein hydrolysis and proteolytic activity was observed with a bacterial isolate ATP-P5 (2.0 IU/ml) Table 3.1.

Identification

After gram's staining, the culture was observed purple colour Gram +ve rods (Fig 3.2), So it was confirmed that the isolated culture was *Bacillus* sp.

Assay of proteolytic activity and protein estimation

Bacterial isolate ATP-P5 was inoculated individually in Glucose yeast casein broth enriched with casein. The inoculated broth was incubated at 50° C under shaking (120 rpm). The cell free broth was harvested and tested for the protease activity at A_{280} (Table 3.2). Specific activity was observed after the protein estimation and enzyme activity. One unit of enzyme activity (U) was calculated as the amount of cell-free supernatant required to increase absorbance at 275 nm by one unit under the assay conditions. Protein content was estimated by method of Lowery's using bovine serum albumin as the standard and found to be 1.5 IU/mg (figure 3.2).

Biofilm formation and quantification

To screen for the efficiency of enzyme in removing the biofilm; the enzyme treatment was given during the incubation, at 45°C.

The cultures of *E. coli* MTCC-1687 and *Pseudomonas mendocina* MTCC-11808 was grown on nutrient agar plates for the detection of biofilm production on Nutrient agar plate (Figure 3.3).

Detection of Biofilm production from the selected organism by different methods

Tube method

By Tube method**,** visible thick film was obtained inside the wall of tube and bottom of the tube (Figure 3.4).

Table.1 Screening of bacterial isolates for protease production

Sr No	Isolates	Zone of clearance	Activity (IU/ml)
1	ATP-P1	+	0.7±0.1
2	ATP-P2	-	0.8±0.3
3	ATP-P3	+	1.8±0.2
4	ATP-P4	-	0.6±0.4
5	**ATP-P5**	+++	**2.0**±0.1
6	ATP-P6	+	0.8±0.2
7	ATP-P7	+	0.5±0.1
8	ATP-P8	-	0.7±0.08
9	ATP-P9	-	0.8±0.2
10	ATP-P10	-	0.2±0.1
11	ATP-P11	++	1.5±0.3
12	ATP-P12	++	0.5±0.1
13	ATP-P13	-	0.4±0.1
14	ATP-P14	+	0.6±0.1
15	ATP-P15	-	0.7±0.1

(- No zone of clearance, + Zone of clearance, ++ Good zone of clearance, +++ Very good zone of clearance)

Table.2 Quantitative analysis of proteolytic bacteria isolated from the soil samples

Organism	Casein Hydrolysis	Activity (U/ml)	Protein (mg/ml)	Specific Activity(U/mg)
ATP-P5	**Positive**	**2.0±0.005**	**0.9±0.001**	**2.0±0.001**

Table.3 Biofilm produced by *E.coli* and *Pseudomonas mendocina* degraded by Protease enzyme

S. No	Bacteria	Treatment A_{660} (Biofilm+Enzyme)	Control A_{660} (Biofilm)
1.	***E.coli***	**0.230±0.001**	**0.369±0.003**
2.	***Pseudomonas mendocina***	**0.120±0.001**	**0.292±0.10**

Fig.1 Showing zone of hydrolysis around the colonies

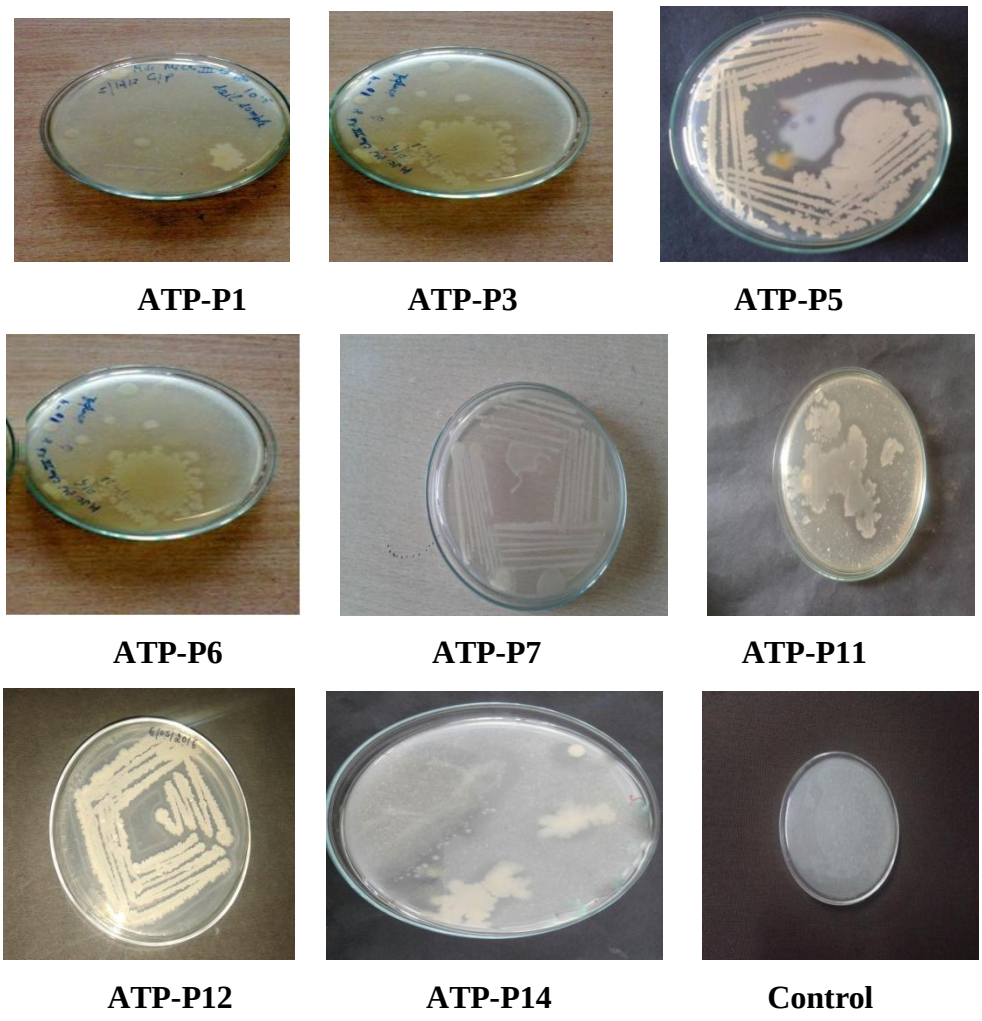


Fig.2 Gram's staining of isolate ATP-P5

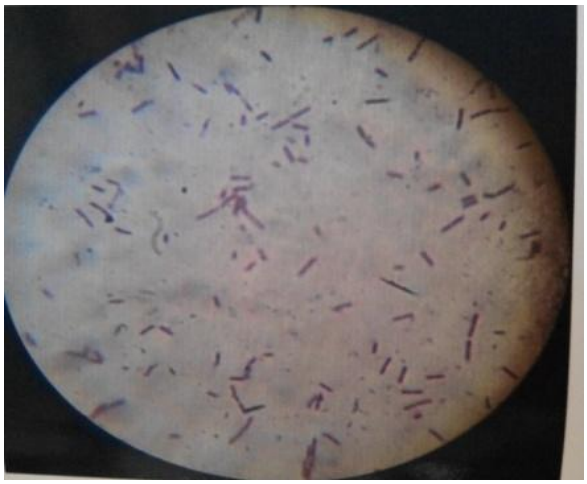
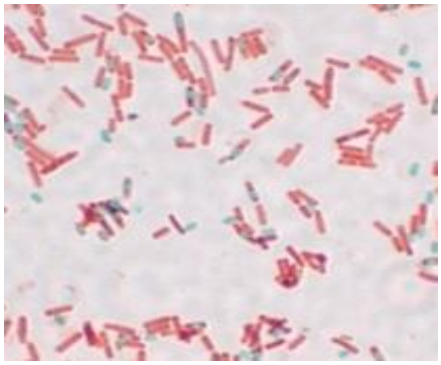
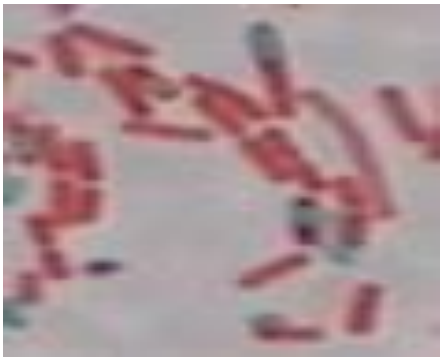


Fig.3 Spore staining of bacterial isolate ATP-P5 by method of Schaeffer and Fulton. Plate A: Presence of endospores (green colored) detected by Malachite green, magnification 1000X; and Plate B: Cropped and magnified view of the bacterial culture showing endospores



(A)



(B)

Fig.4 Lowery's Method

Fig.5 Tube method. Thick film formation gives positive result while thin film formation gives non biofilm producers

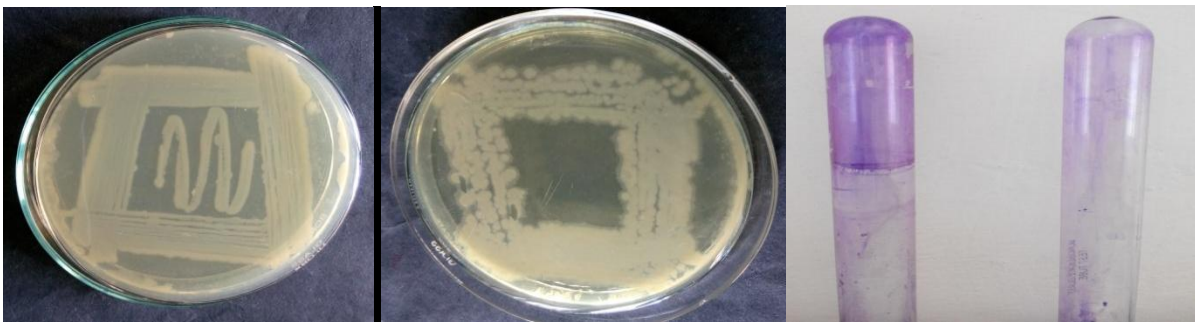
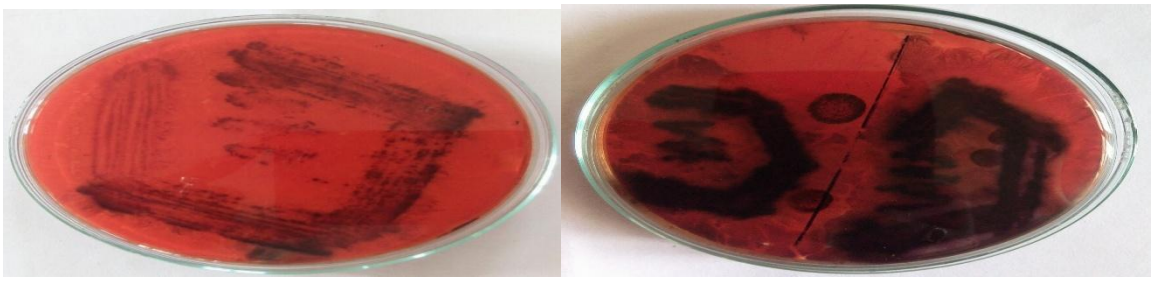


Figure 3.4. Growth of *E.coli* and *Pseudomonas mendocina* on Nutrient agar

Fig.6 Congo red agar method Black colonies shows biofilm formation, while red colonies shows non biofilm producers



Pseudomonas mendocina *E.coli*

Congo red agar method

After using the Congo red in nutrient agar plates**,** black colour colonies were appeared on the nutrient agar plate which sows the presence of biofilm production from the organism (Fig 3.5).

On the congo red agar plate black color colonies shows biofilm formation, while red colonies were non biofilm producers (Figure 3.5). Biofilm formation degraded by using the protease enzyme in the reaction mixture. It is clear from the results (Table 3.3) that when biofilm was treated with the thermophilic protease enzyme the growth of bacteria producing biofilm was minimum (*E.Coli* 0.230 at A_{660} and *Pseudomonas mendocina* 0.120 at A_{660}) as compared to control. Due to significant structural role of protease enzyme, and polysaccharides in the EPS (extracellular polymeric substance) in the matrix of bacteria producing biofilm. The combination of protease and polysaccharides was successful in biofilm removal or detachment of the organisms.

A total of 15 bacterial isolates were isolated from hot water and soil samples and out of these only 8 bacterial isolates hydrolyzed the casein. The maximum casein hydrolysis was observed with a bacterial isolate ATP-P5. There are many reports on isolation of protease producing microorganisms from soil (Mahmoud *et al.,* 2007, Palaniswamy *et al.,* 2008; Kalaiarasi *et al.,* 2009, Ashoken, 2010), tannery waste (Joshi, 2010), industrial effluent (Sangeetha *et al.,* 2008; Feng *et al.,* 2001). Adinarayana *et al.,* (2003) reported quantitative screening of alkalophilic *Bacilli* for protease production by zone of clearance or precipitation of casein around the colonies. Isolate ATP-P5 was catalase, oxidase, lactose, glucose and sucrose positive whereas it was negative for indole test, methyl red test, vogus proskauer, citrate reduction and urease test. These phenotypic characteristics based on Bergey's Manual of Determinative Bacteriology confirmed the isolate ATP-P5 as *Bacillus* sp. This isolate was identified as *Bacillus subtilis* by 16S ribotyping since it showed very close similarity with already existing 16S sequences of *Bacillus subtilis* in the NCBI database. Results of the present study are conformity with the studies of Bajaj *et al.,* 2013; Pravin *et al.,* 2014; Pant *et al.,* 2015 and Patil *et al.,* 2015 they also reported the protease production from *Bacillus* species. However, Gupta *et al.,* 2006 reported protease production from *pseudomonas aeruginosa*. Muthulakshmi *et al.,* 2011 and Chaudhary *et al.,* 2013 reported the protease production from *Aspergillus* species. Selecting the microorganism is important in order to obtain the desired product and its capability to secrete large amount of proteases and it should not produce toxins or any other undesirable products.

Out of so many applications of this useful enzyme as discussed earlier in the literature, the present study has been conducted with the aim to evaluate its application on the biofilm degradation. The biofilms acts as a barrier for antibiotics to affect the pathogenic bacteria and this may account for some people developing chronic Borreliosis/ Lyme symptoms. Currently it is not scientifically known how to permanently degrade the biolfilm,

However it is known that proteolytic enzymes are very effective at dissolving mucupolysaccharides in biofilm. In the present study 0.2 ml of purified protease has been applied for degrading the biofilm of *Pseudomonas mendocina* and *E.coli in* 10 minutes. Similar results had been reported by Bholey *et al.,* (2012) who reported that enzyme of *Bacillus pumilus* and *Staphylococcus auricularis* was capable of degrading 86% and 50% of biofilm respectively. Molobela *et al.,* (2010) used the combined effect of protease and amylase for the degradation of biofilm produced by *Pseudomonas fluorescence* bacteria. Singh *et al.,* (2015) has reported that several fungi have capability to degrade biofilm by secreting various enzymes.

The purified protease was highly compatible with the detergents and help in removing protein based stain from clothes. Purified alkaline protease was also used for recovery of silver from waste X-Ray films and about 0.054g silver was recovered from X-Ray film.

This indicated that alkaline protease from *Bacillus subtilis* has a potential of being applied for recovery of silver from waste X-Ray film as well as for biofilm degradation. In the present study 0.2 ml of purified protease has been used for degradation of the biofilm from *Pseudomonas mendocina* and *E.coli in* 10 minutes.

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